

## Supporting document 1

### Risk and technical assessment report (at Approval) – Application A1096

### Xylanase from *Bacillus licheniformis* as a Processing Aid

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## Executive summary

An Application has been received seeking the permission for a new enzyme for use in the baking industry. This new enzyme is a protein engineered variant of the enzyme, endo-1,4- $\beta$ -xylanase, sourced from a genetically modified strain of *Bacillus licheniformis*.

The stated purpose for this enzyme, namely for use as a processing aid to improve dough handling and characteristics of bread is clearly articulated in the Application. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme preparation meets international purity specifications.

FSANZ's risk assessment concluded that there were no public health and safety issues associated with the use of the enzyme preparation with the commercial name Panzea containing Xyl264 produced by genetically modified *B. licheniformis* strain HyGe329 as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic, pathogenic or sporogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Further, *B. licheniformis* has a history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code.
- Residual enzyme is expected to be present in the final food but would be inactive and susceptible to digestion like any other dietary protein.
- Bioinformatic analysis indicated that Xyl264 has no biologically relevant homology to known protein allergens or toxins.
- Xyl264 caused no observable effects at the highest tested doses in a 90-day toxicity study in rats. The NOAEL was 1020 mg TOS (Total Organic Solids)/kg bw per day, the highest dose tested.
- The Xyl264 enzyme preparation was not genotoxic *in vitro*.
- Orthologous xylanases from a range of other sources have been approved as processing aids by FSANZ.

Based on the reviewed toxicological data, it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment is therefore not required.

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# 1 Introduction

Novozymes Australia Pty Ltd submitted an Application seeking the permission for a new enzyme for use in the baking industry. This new enzyme is a protein-engineered variant of the enzyme, endo-1,4- $\beta$ -xylanase, sourced from a genetically modified strain of *Bacillus licheniformis*. The Applicant claims the purpose of using the enzyme is to improve production processes in the baking industry by facilitating dough handling and improving the characteristics of the final bread.

## 2 Characterisation of the enzyme

### 2.1 Identity of the enzyme

The following information regarding the identity of the enzyme has been taken from the Application and verified from enzyme nomenclature references.

Generic common name	xylanase
Accepted IUBMB <sup>1</sup> name:	endo-1,4- $\beta$ -xylanase
IUBMB Enzyme nomenclature:	EC 3.2.1.8
C.A.S. number:	9025-57-4
IUBMB systematic name	4- $\beta$ -D-xylan xylanohydrolase
Other names:	endo-(1 $\rightarrow$ 4)- $\beta$ -xylan 4-xylanohydrolase, 1,4- $\beta$ -xylan xylanohydrolase,
Commercial name:	Panzea BG and Panzea 10X BG (ten times strength)

### 2.2 Chemical and physical properties

#### 2.1.1 Enzymatic properties

The enzyme catalyses the endo-hydrolysis of the 1,4-B-D-xylosidic linkages in xylans, specifically to modify the arabinoxylans in cereals such as wheat, barley and oats which improves the dough handling and characteristics of the final bread.

The enzyme preparation (granulated powder) is added to the flour or liquid used during the preparation of the dough. The enzyme is active during both the dough preparation and the leavening of the bread. The enzyme is then inactivated due to the high temperatures involved in the bread baking process.

#### 2.1.2 Physical properties

The commercial preparation is supplied as two products of different enzyme activities; one is ten times the strength of the other product.

The two enzyme preparations are provided as granulated off-white powders standardised using wheat flour as the carrier.

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<sup>1</sup> IUBMB – International Union of Biology and Molecular Biology

There are therefore allergen issues for use of the enzyme preparations but since the main use of the enzyme will be in the baking industry this will have no additional allergen impact to those of the other gluten containing ingredients.

## **2.3 Production of the enzyme**

The production of the enzyme is via a submerged fed-batch pure culture fermentation process which is common for the production of many food enzymes.

The production steps can be summarised as a fermentation process, recovery steps to extract the enzyme from the fermentation broth, purification steps and then formulation of the final commercial enzyme preparation. More detail of the individual steps is provided in the Application.

The fermentation process involves two steps, the initial inoculum fermentations to produce enough of the microorganism for the production fermentation and then the main fermentation.

The downstream processing steps taken after the main fermentation to produce the enzyme consist of: killing the production strain (source microorganism), removal of the cell material, ultrafiltration to separate and concentrate the enzyme and finally stabilisation and standardisation using wheat starch to produce the desired enzyme activity in the final enzyme preparations.

## **2.4 Specifications**

The enzyme preparation meets the general enzyme specifications of both the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex. These specification monographs are primary references for specifications in Standard 1.3.4 – Identity and Purity.

## **2.5 Technological function of the enzyme**

The Application contains technical and product data sheets from the Applicant for use and the purported advantages of the enzyme in the baking industry.

Based on the information provided by the company the enzyme provides superior (increased) volume of the baked product, improved crumb texture and appearance, dry, stable dough for better easier handling, high tolerance to variations in flour and process parameters and ease of formulating into flour, bread improvers and premixes. It is further stated that the enzyme is not naturally inhibited by substances commonly found in flour.

Results are presented showing an increase in bread loaf volume of 11% for common CBP (Chorleywood bread process, which is the main bread production process used in Australia and New Zealand) bread, with improved handling and appearance properties. The increase in volume was claimed to be 16% for a mixed wheat/rye loaf, and 35% for French baguettes.

## **2.6 Food technology conclusion**

The stated purpose for this enzyme, namely for use as a processing aid to improve dough handling and characteristics of bread is clearly articulated in the Application. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme preparation meets international purity specifications.

## 3. Hazard assessment

### 3.1 Background

#### 3.1.1 Description of the genetic modification

Xylanase is produced by a GM strain of *B. licheniformis* (production strain HyGe329), which expresses a variant xylanase gene chemically synthesised using, as a basis, a coding sequence in a public database (UniProtKB/TrEMBL #052730 – <http://www.uniprot.org/uniprot/O52730>) for an *xyIY* gene first isolated from *Bacillus* sp.KK-1 and characterised by Yoon *et al.* (1998). The sequence of the XyIY protein is 99% homologous to three other proteins known to come from *B. licheniformis*, one of which is identified as XyIY from *Bacillus licheniformis* WX-02 (<http://www.uniprot.org/uniprot/I0UE70>) and on this basis it is concluded that *Bacillus* sp. KK-1 is *B. licheniformis*.

The synthetic variant xylanase gene has been given the designation *xyI264*. The *xyI264* expression cassette comprises a) a fragment of a hybrid *Bacillus* promoter with promoter elements from *B. licheniformis*, *B. amyloliquefaciens* and *B. thuringiensis*, b) the *xyI264* coding sequence and c) a *B. licheniformis* terminator. The cassette was incorporated into plasmid pBW120 which was then transformed into *Bacillus subtilis* strain BW154. This strain was used as a donor to transfer pBW120 to strain BW302 (see Section 3.2 below) via conjugation. A site-specific integration method mobilised the cassette into two specific loci in BW302. Thus HyGe329 contains two copies of the *xyI264* gene. Sequence information shows there are no other coding sequences from the plasmid present in the final production strain. This means, in particular, there are no antibiotic resistance genes present.

To test the stability of the insert in the production strain, DNA from cells from three separate pilot production batches representing a span of approximately 14 generations was compared to DNA from cells in the master cell bank (MCB). The band patterns obtained for all the pilot batches were identical to the band pattern of the MCB, thus indicating stability of the insert.

#### 3.1.2 Scope of the hazard assessment

The hazard of xylanase derived from *B. licheniformis* strain HyGe329 was evaluated by considering the:

- hazard of the production organism, including any history of safe use in food production processes;
- hazard of the encoded protein, including potential allergenicity; and
- toxicity studies on the enzyme preparation intended for commercial use.

### 3.2 Hazard of the production organism - *B. licheniformis* HyGe329

The production strain HyGe329, containing 2 copies of the *xyI264* gene, is genetically engineered from strain BW302. In turn, BW302 has been derived from a natural isolate of *B. licheniformis*, ATCC 9789, through a series of targeted recombination events to inactivate genes encoding several proteases and unwanted peptides and the *spoIIAC* gene essential for sporulation. The Applicant claims that these modifications enhance the safety and stability of the xylanase that is produced. Additionally there has been insertion of integrases<sup>2</sup> to target the two site-specific integration sites of the *xyI264* cassette into the *B. licheniformis* BW302 genome.

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<sup>2</sup> An integrase is an enzyme that is used to splice DNA into a genome at a specific location

Virulence is not generally associated with *B. licheniformis*, asporogenic forms of which are designated as Risk Group 1 (RG1) - agents that are not associated with disease in healthy adult humans (NIH, 2013). There are, however, strains of *B. licheniformis* that have been implicated in human infection in immunocompromised individuals and neonates (EPA, 1997). Toxin-producing isolates of *B. licheniformis* have been isolated from raw milk, commercially-produced baby food and other foods involved in food poisoning incidents. However, pathogenicity has been restricted to severely immunocompromised patients.

*B. licheniformis* is widely used to produce food-grade enzymes. FSANZ has previously assessed the safety of *B. licheniformis* as the source organism for a number of food processing aids and the following enzymes derived from *B. licheniformis* (both GM and non-GM) are listed as permitted in Standard 1.3.3 of the Code:  $\alpha$ -amylase, maltotetrahydrolase, pullulanase, glycerophospholipid cholesterol acyltransferase, and serine proteinase.

Data submitted with the application indicated that *B. licheniformis* HyGe329 is not detectable in the final enzyme preparation to be used as a food processing aid. The organism is removed during a multi-step recovery of the purified enzyme following submerged fed-batch pure culture fermentation. The final stage involves filtrations at defined pH and temperature intervals that result in an enzyme concentrate solution free of the production strain.

The USFDA has granted GRAS to the enzyme preparation under the intended conditions of use (U.S.FDA, 2013).

### **3.3 Hazard of the encoded protein - xylanase**

Xylanases are widely distributed, occurring in diverse genera of bacteria, actinomycetes and fungi (Beg *et al.*, 2001). They have been used for several decades in the food industry (Pariza and Johnson, 2001) including in baking (particularly bread-making) and fruit juice and beer clarification (Sharma and Kumar, 2013). Xylanases from a number of sources have been approved as processing aids by FSANZ.

Xyl264 is a protein that differs from the native XylY protein by a single amino acid residue. The Applicant states that the additional amino acid in XylY protein alters the specificity of the enzyme and therefore increases the production of oligosaccharides in, and hence reduces the stickiness of, dough treated with Xyl264. An amino acid database search of naturally occurring xylanases within the GH8 family of glycoside hydrolases revealed that there is a high level of variation with the sequence similarity in some cases being as low as 40%. The Applicant also provided bioinformatic information showing that single amino acid additions of the type introduced in Xyl264 can occur naturally in some enzymes. However, there are no data currently available to confirm that naturally occurring xylanase contains this additional amino acid.

The intention is that the enzyme preparation (comprising wheat flour, sodium chloride and Xyl264, formulated to achieve the desired enzyme activity – see Table 1) is added to baking ingredients. The enzyme is active during dough preparation and leavening but is inactivated at the high temperatures used during baking. It is likely that any residual enzyme in the final food would therefore be present as denatured protein and undergo normal proteolytic digestion in the gastrointestinal tract. To confirm the digestibility of Xyl264, potential cleavage sites were investigated by FSANZ using the amino acid sequence of Xyl264 and the PeptideCutter tool in the ExPASy Proteomics Site ([http://web.expasy.org/peptide\\_cutter/](http://web.expasy.org/peptide_cutter/)). Xyl264 has multiple cleavage sites for pepsin (78 sites at pH 1.3 and 134 sites at pH >2), trypsin (46 sites), chymotrypsin (56 high-specificity sites, 106 low-specificity sites) and endopeptidases (73 sites). On this basis, Xyl264 is considered likely to be as susceptible to digestion as the vast majority of dietary proteins.

Bioinformatic analyses were performed to assess the similarity to known allergens and toxins of the Xyl264 protein.

### 3.3.1 Bioinformatic analyses for potential allergenicity

The *in silico* analyses compared the Xyl264 sequence with known allergens in two datasets:

- the FARRP (Food Allergy Research and Resource Program) dataset within AllergenOnline (University of Nebraska; <http://www.allergenonline.org/>).
- the dataset within the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. (<http://www.allergen.org/>).

Three types of analyses were used to search the databases:

- a) the FASTA algorithm (Pearson and Lipman, 1988) version 3.4, using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). The E-score<sup>3</sup> generated by this indicates whether there are any alignments that meet or exceed the Codex Alimentarius (Codex, 2003) FASTA alignment threshold (35% identity over 80 amino acids) for potential allergenicity. This threshold aims to detect potential conformational IgE-epitopes.
- b) The same as a) but with scaling enabled in order to find any matches that may have high identity over windows shorter than 80 amino acids.
- c) The Needleman-Wunsch global alignment algorithm (Needleman and Wunsch, 1970) in the program package EMBOSS. (<http://www.ebi.ac.uk/Tools/emboss/>). This explores all possible alignments and chooses the best one (the optimal global alignment). Hence it can identify those hits with more than 35% identity over the full length of the sequences.

No significant homology with any known allergens was determined. The conclusion from these bioinformatic analyses is that Xyl264 does not show biologically relevant homology to any known allergen and on this basis is unlikely to be allergenic.

### 3.3.2 Bioinformatic analyses for potential toxicity

The Xyl264 sequence was compared to sequences present in the UniProtKB database (<http://www.uniprot.org/>) containing entries from Swiss-Prot and TrEMBL and using the term 'toxin' to refine the search. The comparison method used a ClustalW 2.0.10 sequence alignment program (<http://www.clustal.org/clustal2/>) (Larkin *et al.*, 2007) to align each sequence from the database with the Xyl264 sequence.

The greatest amino acid homology found was only about 16% with proteins known to be toxins. This low level of homology indicates that Xyl264 is unlikely to be toxic.

## 3.4 Evaluation of unpublished toxicity studies

### Report submitted

Ravn, B.T.; Pedersen, P.B. (2013). Summary of toxicity data. Xylanase, PPQ33502 from *Bacillus licheniformis*. Report ID # 2013-03354-01, Novozymes A/S (unpublished).

<sup>3</sup> Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Commonly, for protein-based searches, hits with E-values of  $10^{-3}$  or less and sequence identity of 25% or more are considered significant although any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis, 2005).



Unpublished toxicity studies on the Xyl264 preparation were submitted by the Applicant and independently evaluated by FSANZ. These studies comprised two genotoxicity test (Ames test and an *in vitro* micronucleus assay) and a sub-chronic toxicity study in rats. The test substance was Xyl264 (designated as batch PPQ33502) prepared to the manufacturer's specifications. The test substance was supplied in liquid form (dissolved in water) and differed from Panzea 10X BG which would normally be supplied as granules. A comparison of PPQ33502 and Panzea X10 BG is given in Table 1.

**Table 1: Comparison of PPQ33502 and Panzea X10 BG**

Characteristic	PPQ33502	Panzea X10 BG
Activity	3670 NXU/g	2350 NXU/g
Water (% w/w)	88.3	-
Wheat Flour (% w/w)	0	90
Sodium chloride (% w/w)	0	6
Ash (% w/w)	2.0	6
Total Organic Solids (% w/w)	9.7	4

### 3.4.1 Genotoxicity

#### Individual studies

Pedersen, P.B. (2012). Xylanase PPQ33502: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. Study ID # 2012-13362-01, Novozymes A/S (unpublished).

[The study contained a statement of compliance with principles of GLP and a quality assurance statement. It was conducted in accordance with OECD Test Guideline 471 (OECD, 1997)].

Whitwell, J. (2012). Xylanase PPQ33502: Induction of micronuclei in cultured human blood peripheral blood lymphocytes. Reference # 20126017, Covance (unpublished).

[The study contained a statement of compliance with principles of GLP and a quality assurance statement. It was conducted in accordance with OECD Test Guideline 487 (OECD, 2010)]

The results of these *in vitro* studies are summarised in Table 2. Negative (water vehicle) and positive controls were tested in each study and gave expected results. It is concluded that the Xyl264 preparation did not induce gene mutations or show any clastogenic activity.

**Table 2: Summary of genotoxicity test results**

Test	Test system	Test article	Concentration or dose range	Result
Bacterial reverse mutation (Ames test)	<i>Salmonella typhimurium</i> strains TA 98, 100, 1535 & 1537 <i>Escherichia coli</i> strain WP2 <i>uvrA</i>	Xyl264 obtained from <i>B. licheniformis</i>  (Batch No.PPQ33502); 4% TOS  Water vehicle	156 – 5,000 µg dry matter/mL	Negative (±S-9) <sup>1</sup>
<i>In vitro</i> micronucleus assay	Human lymphocytes	As above	500 – 5000 µg/mL	Negative (±S-9) <sup>1</sup>

S-9 = metabolic activation system comprising liver preparation from rats induced with Aroclor 1254

### 3.4.2 Subchronic toxicity study

#### Individual study

Homstrøm, M.W. (2013). Xylanase PPQ33502: A 90-day gavage toxicity study in rats. Reference ID # 20126010, CiToxLAB Scantox A/S (unpublished).

[The study contained a statement of compliance with principles of GLP and a quality assurance statement. It was conducted in accordance with OECD Test Guideline 408 (OECD, 1998).]

The Xyl264 preparation (Batch No. PPQ33502) was administered by gavage to four groups of 10 SPF Sprague Dawley (strain Ntac:SD) rats/sex at doses of 0, 102, 336 or 1020 mg TOS/kg bw/d for 90/91 days (water vehicle). Rats were sourced from Taconic Europe A/S (Ejby, Denmark), were approximately 5 weeks old and had body weights within a range of  $\pm$  20 g for each sex at the commencement of dosing. Rats were housed under standard conditions, with food and water available *ad libitum*. Standard gross toxicological endpoints were recorded during the treatment period (deaths, clinical signs, bodyweight and food consumption). Ophthalmoscopy was performed prior to treatment and on day 90. Pre-treatment and before termination, the animals were examined with respect to motor activity (open field test) and reactivity to different types of stimuli. Blood was collected on day 91 for the analysis of standard haematology and clinical chemistry parameters. Urine was collected prior to sacrifice for analysis of standard urinary parameters. Following sacrifice, rats were necropsied, organ weights recorded and standard tissues prepared for histopathology.

There were no deaths, clinical signs, motor activity abnormalities or ophthalmic abnormalities attributable to administration of the test substance. Bodyweight gain and food consumption were comparable across all groups. There was no treatment-related effect on any haematology, clinical chemistry or urinary parameter. There were no treatment-related macroscopic abnormalities, differences in organ weights or histopathological findings. The NOAEL (No Observed Adverse Effect Level) for both males and females was 1020 mg TOS/kg bw/d, the highest dose tested.

### 3.5 Hazard assessment conclusions

There are no public health and safety issues associated with the use of Panzea containing Xyl264 produced by GM *B. licheniformis* strain HyGe329 as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic, pathogenic or sporogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Further, *B. licheniformis* has a history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code.
- Residual enzyme is expected to be present in the final food but would be inactive and susceptible to digestion like any other dietary protein.
- Bioinformatic analysis indicated that Xyl264 has no biologically relevant homology to known protein allergens or toxins.
- Xyl264 caused no observable effects at the highest tested doses in a 90-day toxicity study in rats. The NOAEL was 1020 mg TOS/kg bw per day, the highest dose tested.
- The Xyl264 enzyme preparation was not genotoxic *in vitro*.

- Orthologous xylanases from a range of other sources have been approved as processing aids by FSANZ.

Based on the reviewed toxicological data, it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment is therefore not required.

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